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Characterisation of the Adhesive Proteins of a

Major Fouling Organism, the Barnacle.

Michael J. Naldrett

US Army RD & E Center, Kansas St,

Natick, MA 01760, USA

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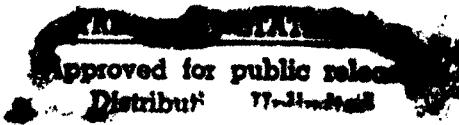
ABSTRACT

Solidified adhesive (cement) of the balanid barnacle, *Balanus crenatus* (Crustacea: Cirripedia) was dissolved using sodium dodecyl sulphate with and without the addition of the reducing agent, 2-mercaptoethanol. The protein(s) which makes up the cement is heat sensitive demonstrating its hydrophobic nature. Other problems with the dissolution of the cement are due to the abundant sulphur cross-links which appear to be buried in hydrophobic cores and are inaccessible to the reductant. Dissolution of the cement was achieved by grinding the wet cement in water to produce a fine suspension, adding a buffer with SDS (4 %) and allowing to stand at no higher than 60 °C for a minimum of 30 min. (optimum temperature = 40 °C). Non-reducing SDS-PAGE revealed numerous bands ranging in size from 2 to 46 kD which were reduced to a minimum of 2 bands of roughly 2.5 and 3 kD. These proteins were blotted from SDS-PAGE tricine gels onto polyvinylidene difluoride membrane for modification, amino acid analysis and sequencing.

INTRODUCTION.

Major interest in the adhesive (commonly called cement) used by adult barnacles first started over 20 years ago. Its possible application as a dental restorative agent (*e.g.* Jefferson-Bowen *et al.*, 1974) never developed because the material defied complex analysis. However, barnacle cement still represents one of the few adhesives which can stick surfaces together underwater, a problem which the inventors of synthetic adhesives continue to struggle with. Furthermore, a complete understanding of how this glue works is also of key interest to those working in the field of anti-fouling.

With the cement, the barnacle can stick to almost any surface it encounters in the marine environment making this animal a formidable fouling organism. In today's world of high oil prices the most significant economic consequence of barnacle fouling is in the cost of fuel oil for the propulsion of ships. Barnacle fouling causes a significant increase in the roughness of the underwater hulls of ships and raises fuel consumption by as much as 40 %. This is not surprising when one considers that an increase of hull roughness of some 10 μm can increase fuel consumption by 1 % (Lackenby, 1962).

Fuel penalties are not the only cost to be taken into account. To realise the extent of the problem we must consider the costs of underwater cleaning (or in some cases dry-docking the vessel) as well as the costs of anti-foulant paints and time. To give some idea of the costs involved, the value of cleaning North Sea oil platforms alone in 1977 was reported at £3m with a projected estimated value of between £15m and £24m by the mid-1980s (D.O.E. report 1980). Include shipping, sea water cooling systems and the cost due to increased corrosion of submerged structures and the importance of the adhesive used by barnacles immediately becomes apparent.

The analysis of barnacle cement started during the late 60's and early 70's (see review Yule & Walker, 1987) however only partial characterisation was possible because of the cement's resistance to chemical denaturants. Histochemical (Walker, 1970) and amino acid analyses (see Naldrett, 1992 p. 156) showed that barnacle cement contained protein, the exact proportion of which was shown to lie in the region of 90 % (Walker, 1972; Lindner & Dooley, 1973; Yan & Tang, 1981)

The suggestion that barnacle cement contains tanned protein and hence quinones has recently been challenged and instead the view that cement is a collection of hydrophobic proteins held together by sulphur cross-links has been put forward (Naldrett, in press). This report advances those initial observations using cement from *Balanus crenatus*, (probably the most insoluble from of barnacle cement found so far). The way forward for the complete characterisation of this cement is set out along with some possible uses of the material.

MATERIALS AND METHODS

There are two types of barnacle cement which can be most usefully described as primary cement (produced whilst the animal is naturally attached to a substratum) and secondary cement (produced in response to the animal being dislodged from its substratum). Only primary cement was used in this study. It is now thought that there is no difference between primary and secondary cement (Lindner & Dooley, 1973), but the terminology first used by Saroyan et al. (1970) remains useful in abbreviating how cement has been collected.

Collection of primary cement.

Fresh *Balanus crenatus* Bruguière were obtained in early October, 1990 from Newton Ferrers, Devon, England. One year old individuals were collected from metal panels coated with a silicone-based anti-fouling paint, Intersleek™ (International Paint, Gateshead). Barnacles were cleaned of debris by vigorous brushing with a toothbrush in copious quantities of sea water. Once most of the shell had been cleaned each animal was rebrushed quickly in running deionised water. The cement layer was then cut away using a razor blade. All the cement was pooled and stored wet at - 20 °C until needed.

Dissolving Cement

Treatment 1: Finely ground cement was soaked in SDS-PAGE reducing sample buffer (4 % SDS, 5 % 2-ME) for 92 h at 45 °C and was heated to 100 °C for 5 min prior to loading onto the gel.

Treatment 2: Finely ground cement was placed in SDS-PAGE reducing sample buffer (4 % SDS, 5 % 2-ME) and boiled for 20 min. It was then left at 25 °C for 72 h and then heated to 100 °C for 2.5 min.

<u>Treatment 3</u>: Cement was ground more finely than in treatments 1 & 2 by the addition of silica beads to the tissue grinder. Both reducing and non-reducing sample buffers were added and the samples boiled for 1.5 min.

<u>Effect of heat and exposure time on cement placed in SDS + 2-ME.</u>

Of all the denaturants so far used to dissolve barnacle cement, a combination of SDS + 2-ME has proven most successful (Barnes & Blackstock, 1976; Yan & Pan, 1981; and Naldrett, 1992; *in press*). However, temperature and particle size of the cement are also key factors controlling its solubility. For this reason aliquots of cement were analysed by gel electrophoresis after the following treatments:

CONDITIONS (Reducing/ Non-reducing)	INCUBATION	
	time	temp ($^\circ$C)
	15 h	25
	15 h	40
Finely ground cement + sample buffer (SDS + 2ME)	5 h	20
	then 3 min	100
	3 min	100

<u>S.D.S.-P.A.G.E.</u>

Electrophoresis was performed using a Novex mini gel system (Novex Corp, Encinitas, California) with precast gradient slab gels (10-20 % acrylamide; dimensions: 180 x 160 x 1 mm) and premade buffers. This tricine-SDS discontinuous buffer system is based on

that described by Schägger & Von Jagow (1987). The following carboxymethylated rainbow molecular weight standards (Amersham Corp, Illinois) were run on all gels alongside the uncharacterised samples: myosin (200 kD); phosphorylase b (97.4 kD); bovine serum albumin (69.0 kD); ovalbumin (46.0 kD); carbonic anhydrase (30.0 kD); trypsin inhibitor (21.5 kD); lysozyme (14.3 kD); aprotonin (6.5 kD); insulin (b) chain (3.4 kD); and insulin (a) chain (2.35 kD). To aid dissolution, the cement was mechanically ground into fine particles using a ground glass tissue grinder before the addition of sample buffer.

Following electrophoresis, the gels were stained either with 0.1 % Coomassie Brilliant Blue in a methanol, glacial acetic acid, water mix (5 : 1 : 5), or fixed in a methanol: glacial acetic acid: water mix (4 : 1 : 5) before silver staining (Wray *et al.*, 1981).

Electroblotting of Cement Proteins

Proteins separated by gel electrophoresis using the above system were wet-blotted onto a polyvinylidene difluoride (P.V.D.F.) membrane (Immobilon™, Millipore) using a carbonate buffer made up as follows: 3mM sodium carbonate, 10 mM sodium bicarbonate, pH 9.9 in 10 % methanol. Blotting was carried out for 1.5 h at 30 volts constant voltage before the membranes were removed and rinsed in deionised water. Protein bands were then made visible by staining with 0.1 % w/v amido black-10B in 10 % (v/v) methanol, 2 % acetic acid.

Modification of Cysteine Residues.

To prevent cysteine residues from being oxidised during protein sequencing or amino acid analysis this amino acid can be modified to produce stable derivatives. Carboxymethylation of microgram quantities of protein is carried out using iodoacetamide. Alkylation is a convenient alternative for allowing the quantitation of

cysteine and uses the reagent 4-vinylpyridine to produce *S-P-*(4-pyridylethyl)cysteine (PEC). The method used for both modifications was carried out as described by Charbonneau (1989).

<u>Amino acid analysis</u>

Samples of primary cement of *B. crenatus* were dialysed extensively against deionised water primarily to remove sodium chloride. All the material was pooled and freeze-dried. Approximately 1 μg of freeze-dried cement and 5 μl of water was placed in a glass 6 x 50 mm tube. The samples were dried and 200 μl of constant boiling HCl was added with 3-5 crystals of phenol. The samples were then hydrolysed *in vacuo* and under nitrogen for 24 h at 112 $^{\circ}$C. Amino acids were derivatised with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids which were analysed and quantitated on a Pico-Tag $^{\circledR}$ Work Station with 15 cm column (Waters, Milford, MA).

RESULTS.

The cement of *Balanus crenatus* has been analysed previously using Laemmli (1970), tris-glycine polyacrylamide gels (Naldrett, 1992, *in press*). The result of running identical samples on tricine gels has revealed that the cement is very sensitive to the conditions under which it is analysed. In Table 1 a comparison is made of the main molecular weights which appear under both systems using samples of cement prepared for analysis in the same way. It should be noted however, that in all cases so far recorded many minor bands also stain up to give a strong background to the major bands.

Table 1: A comparison of the major cement proteins seen on two different gel systems (tris-glycine and tricine buffer systems) when the cement is treated in exactly the same way.

Molecular Weights of major bands appearing on different types of SDS-PAGE gels	
Tris- Glycine	Tricine
84	78
47	61
39	27
15	24
<15 (unresolved)	

Table 1 shows that both types of gel electrophoresis give completely different results. Furthermore, the electrophoretograms of cement run on tricine gels under reducing conditions differ depending on the treatment of the cement sample prior to loading (Table 2).

Table 2: The molecular weights of the major cement proteins appearing upon gel electrophoresis using 3 different treatments of the same cement.

Treatment 1	Treatment 2	Treatment 3 (NB very finely ground).	
Reducing	Reducing	Reducing	Non-reducing
Molecular Weight (kD)			
			118
85	86	85	
			72
62			56
	49		
	36		
30	30		
	19.5		
	13	13	
	10	10	11

Using treatment 3, cement was found to dissolve even in non-reducing sample buffer illustrating the presence of hydrophobic interactions. Sulphur cross-links are also known to exist and it has been suggested (Naldrett, *in press*) that the smallest proteins seen on gels may well be the cement monomers, all other bands being incompletely reduced complexes of these smaller units. Both these factors fitted the observation that the solubility of ground up cement was highly dependent on its particle size. There was also some suggestion from the data that heating the cement for prolonged periods resulted in the formation of higher molecular weight bands on the gels. As a result of those observations the cement was subjected to varied heat and exposure time conditions. The results of these experiments are shown in Plate 1. The proteins, of what is probably the most insoluble type of barnacle cement, are clearly illustrated. The cement, when ground up finely enough, dissolves even under non-reducing conditions. Gel electrophoresis of this material revealed that the cement contains a range of molecular weights spanning from 2.5 to 46.0 kD and which seem to be multiples of a peptide which has a molecular weight of 2.5 kD. When this non-reduced material was heated at a temperature of 60°C or higher bands with molecular weights as high as 200 kD in size appeared. Also the cement which was not soaked before being heated to 100 °C in sample buffer showed no bands less than 11 kD and did not dissolve as well as those samples which were soaked. It seems that even before accounting for the action of sulphur cross-links we must recognise the presence of strong hydrophobic interactions which occur between the proteins. These, just as with many membrane proteins, cause the proteins to form aggregates on heating, a factor which is not favourable for the dissolution of the cement.

Separation of the reduced samples showed that barnacle cement does indeed consist of multiples of a very small subunit (Plate 1). Using a low temperature reduces the
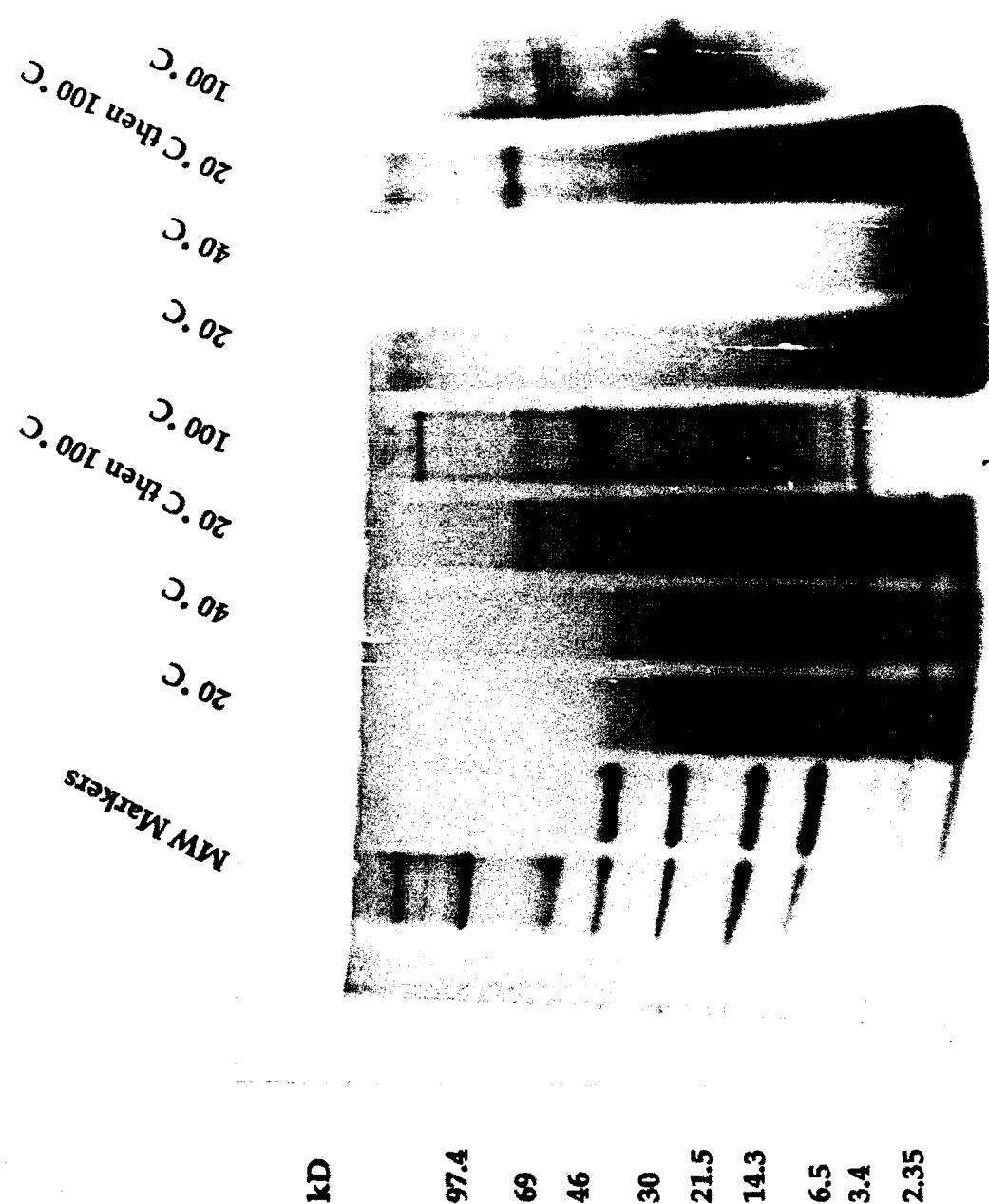
Plate 1   The adhesive proteins of a balanid barnacle (*Balanus crenatus*) showing their sensitivity to heat. Analysis was carried out by SDS-PAGE using a tricine buffer system. Proteins were stained using Coomassie.

hydrophobic element lessening the formation of complexes. The SDS then sufficiently denatured the remaining complexes and allowed the reductant to reach the disulphide bonds which would normally be buried in a hydrophobic environment. In this way the whole matrix was reduced to one or two bands of less than 3.5 kD. However the "20°C + 100 °C" reduced track in Plate 1, shows that should the temperature rise above 60 °C these once completely reduced samples start to reform into complexes. Therefore for SDS-PAGE to be used successfully for the analysis of barnacle cement the boiling step prior to loading the samples onto the gel should be removed and instead the samples should be left at 40 °C overnight, or at least for a minimum of 2 hours. The minimum number of bands so far seen in barnacle cement is two (2.5 & 3 kD). However, even with the resolving power of a tricine gel, which can detect proteins of 1 kD, it is hard to be sure using electrophoresis alone whether or not the smallest band seen on the gels is a single protein.

Amino acid analysis of the bulk cement carried out using the Picotag® system does not compare well with the previous analysis of the same sample. Both are given in Table 1. The analysis of the bulk cement suggests that no one amino acid is more abundant than any other. Cysteine however, has been detected at levels up to 9 % in the cement of *B. crenatus* though in the analysis here most has been destroyed by oxidation during hydrolysis. Attempts to quantitate the CYS residues by modifying the 2.5 kD protein whilst on PVDF membrane has failed so far. However, from the analysis of bulk cement a theoretical isoelectric point (pI) of 9.96 has been calculated.

Table 3. The amino acid compositions (residues/100 residues) of the primary cement of *B. crenatus*.

Residues/100	Cement from *Balanus crenatus*	
	Naldrett (*in press*)	Picotag analysis
ASP	7.32	9.92
GLU	7.36	9.15
SER	8.27	8.62
GLY	7.97	8.41
HIS	2.45	2.93
ARG	6.91	8.12
THR	5.00	6.44
ALA	5.94	5.53
PRO	4.12	3.92
TYR	5.96	6.16
VAL	6.01	5.62
MET	1.21	0.57
CYS	8.56	3.02
ILE	4.48	3.96
LEU	8.11	7.29
PHE	4.54	4.07
LYS	5.80	6.27
TRP	0.00	0.00

DISCUSSION.

The most recent literature on the cement of balanid barnacles revealed that quinones and quinone cross-links are absent from cement (Naldrett, *in press*). Instead, the involvement of hydrophobic interactions and sulphur cross-links was suggested to govern the polymerisation of the cement. The few works on barnacle cement which tried to separate the proteins in the matrix suggested that many sizes of protein were present, not unlike the initial SDS-PAGE analyses presented here. The work carried out by Naldrett (*in press*) on 3 different balanid species revealed two common features. All cements contained a 39 kD protein and a group of proteins which ran at molecular weights of 15 kD and below. These were considered to be the smallest units of the cement. Other protein bands appeared on the gels but never with any consistency and so were considered to be complexes which had not been fully broken down. The results presented here reveal that the multitude of bands which had been detected previously were in fact complexes which can be reduced to a possible minimum of two bands. However, there may be more than two proteins because the resolving power of SDS-PAGE is at its limit for this size of protein. To check if there is more than one protein of this size some other chromatographic technique will have to be employed.

One of the most puzzling features of cement has finally been resolved. Every time cement was dissolved in SDS + 2-ME and separated by SDS-PAGE a darkly staining background of proteins appeared behind the major protein bands. Using tricine gels this background blur was resolved revealing proteins of many sizes. Possible reasons for the background were attributed to bacterial or enzymic digestion; cleavage during boiling; and partial destruction and/or reformation of complexes. Now that cement has been dissolved and analysed in a non-reducing buffer it is clear that the small 2.5 to 3 kD proteins polymerise through sulphur cross-links to give a wide range of polymer sizes. These are resolved clearly on tricine gels. However, when incomplete reduction occurs

each of these bands becomes broader and they merge to give a continuous faint background. This disappears only when complete reduction occurs.

Gel electrophoresis has clearly illustrated the presence of both sulphur cross-links and hydrophobic interactions. The discovery of a darkly staining 2.5 kD protein suggests that this is the major cement protein. Despite being predicted to be only about 25 residues long an initial amino acid analysis of this single band suggests that all amino acids except tryptophan are present.

Despite the apparent simplicity of the cement, its analysis presents some difficulties for the simple reason that it is only soluble in the detergent SDS - very little goes into solution in 8 or 10 M Urea. So far it has been possible to blot cement proteins onto a PVDF membrane from where it should be a simple matter to sequence them. Being only 20-30 amino acids long it is feasible that the full sequence could be obtained without the need for digesting the protein. The trouble comes with confirming any blank spots in the sequence *ie* the amino acids which have been oxidised by the Edman chemistry. The most useful way would be to pyridylethylate the protein on the blot and then sequence thise. It is possible however, that once the protein is blotted and the SDS removed the cysteine residues will once again become unavailable for modification. Indeed this seems to have occurred in the attempts to modify the samples prior to amino acid analysis. No attempt has been made to modify the protein in SDS since this is not a textbook experiment, though both SDS and acetonitrile may be feasible solvents. Another method to analyse the blanks in sequence data would be to cleave the protein with cyanogen bromide leaving peptides which may then be more soluble in urea or even water. These peptides could then be modified and then purified by reverse phase chromatography before sequencing the peptides. Fragments could then be matched up with the previously sequenced protein to fill in the picture.

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